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The Dichotomous Functions of Passenger Leukocytes in Solid-Organ Transplantation

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THE CONCEPT OF PASSENGER LEUKOCYTES AND THEIR ROLE IN REJECTION

Leukocytes or hematolymphoid cells reside, at least temporarily, in the interstitium of all organs. These cells are known as "passenger leukocytes" when the organ becomes an allograft, because they are carried along with the parenchyma and stroma into the body of the recipient. Passenger leukocytes are the most immunogenic cells in an allograft and function as initiators of the rejection reaction.[1-3] Steinman and colleagues[4-8] identified a unique cell type that has become the passenger leukocyte prototype, the interstitial dendritic cell (DC). It is characterized by intense major histocompatibility complex (MHC) class II antigen, costimulatory[9] and adhesion molecule expression,[10, 11] and is the most effective (and perhaps only) stimulator of a mixed lymphocyte reaction (MLR).[8]

Besides DCs, allografts contain many other types of donor leukocytes. Their number and phenotypic composition vary with the organ. For example, intestine and lung allografts are "leukocyte-rich," and a high percentage of the cells are mature T and B lymphocytes. Liver allografts are

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also considered leukocyte-rich, but contain more macrophages, B cells, and hematolymphoid progenitor cell populations. By comparison, heart and kidney allografts could be considered "leukocyte-poor," but these organs still contain an appreciable number of DCs.

Since cells normally traffic into and out of organs, some passenger leukocytes will emigrate from an allograft into recipient tissues immediately after transplantation, while others remain in the allograft. This explains the finding of donor leukocytes in the recipient spleen within hours to days after transplantation.[12–14] Unless the donor and recipient are HLA-identical, arrival of the donor leukocytes (especially the DCs) in the recipient spleen[12–14] and arrival of recipient lymphocytes in DC-rich areas of the allograft[14, 15] elicit the in vivo equivalent of the in vitro MLR.[12–14] The former is known as "central sensitization" and the latter as "peripheral sensitization." Both are initiation sites of the rejection reaction that is ultimately directed at the parenchyma and blood vessels of solid-organ allografts.

The scenario just described (i.e., that passenger leukocytes initiate rejection) has guided the field of transplantation biology for the last 25 years. Investigators have therefore attempted to purge passenger leukocytes from donor organs prior to implantation in the expectation of prolonging graft survival, or achieving graft acceptance without immunosuppressive therapy.[16] Although this approach has clearly resulted in improved short-term allograft survival, the allografts depleted of passenger leukocytes are still eventually rejected. This finding is consistent with the observation that even pure keratinocyte cultures, devoid of all contaminating passenger leukocytes, are rejected.[17] Moreover, even after the passenger leukocytes are destroyed, the allograft is still susceptible to chronic rejection.[18] These observations suggest that the above model cannot adequately explain some fundamental observations in transplantation biology.

Batchelor and Braun[19] have more fully developed the above paradigm to explain this discrepancy. They suggest that *acute rejection* is mediated by a distinct set of alloreactive recipient T cells that are *directly* activated after physical contact with donor DCs. The reaction observed in vivo is akin to the in vitro MLR. As mentioned above, it occurs both within the allograft and in the recipient's lymphoid tissue. It manifests as a burst of immune activation, usually occurring during the first few weeks after transplantation. In the allograft this reaction is also known as acute cellular rejection, and for the most part is easily controlled with increased immunosuppression. A period of relative calm follows, when the donor passenger leukocytes within the allograft are replaced by recipient cells. The donor cells released from the allograft are thought to be destroyed, or simply die out.

Unfortunately, another distinct set of alloactivated T cells emerges that mediate chronic rejection.[19] *Host-derived* antigen-presenting cells process and *indirectly* present to the recipient immune system donor MHC and possibly other antigens shed from the allograft. Theoretically, this shedding of foreign epitopes subjects the allograft to indolent but relentless low-grade damage that eventually results in the development of obliterative arteriopathy, or chronic rejection. If this form of rejection

could be avoided, organ transplantation would become more routine than it already is, because a continual decline of allograft function plagues most long-term survivors of kidney and heart transplantation, but liver allografts are relatively resistant. Later, we explore a possible relationship between passenger leukocytes and hepatic resistance to chronic rejection.

DONOR BONE MARROW CELLS AS TOLEROGENS

In a separate area of transplantation biology research, investigators have shown that donor hematolymphoid cells carry with them the ability to render the recipient's immune system specifically unresponsive to subsequent allografts from the same donor. This concept was first uncovered by Owen[20] in twin cattle fetuses that shared placental circulations. The immune systems of the twins were naturally mixed chimeras, or composed of hematolymphoid cells from both individuals. They were able to accept organ allografts from each other without the need for immunosuppression. Billingham, Brent, and Medawar[21] and Main and Prehn[22] later achieved the same results in adult rodents, by infusing donor bone marrow into a recipient whose own immune system had been destroyed by irradiation. These animals had fully chimeric immune systems, composed exclusively of donor hematolymphoid cells, and they accepted other organs from the same donor without immunosuppression. Unfortunately, attack of the defenseless recipient by the donor hematolymphoid cells,[23] or graft-versus-host disease (GVHD), has limited the clinical applicability of this approach.

Because of the early promise of subsequent drug-free allograft acceptance, numerous subsequent experimental animal models have pursued variations of the irradiation–donor bone marrow infusion protocol. Chemotherapeutic cytoablation and monoclonal antibody therapy with lower-dose or localized irradiation, and donor bone marrow infusion protocols were successfully developed.[23–35]

One variation of the induced chimerism approach that has been particularly successful in small animals was developed by Ildstad and Sachs.[36] It also illustrated an important concept. They used conditioning irradiation followed by a *mixture* of both donor and recipient bone marrow cells that routinely resulted in *mixed* allogenic chimeras. These animals were both tolerant of donor allografts *and* resistant to GVHD.[36] Despite mixed chimeric immune systems, allogenic cell populations were able to cooperate and the animals remained healthy and apparently not susceptible to disease. More than anything else, the studies just cited have shown that specific allogenic tolerance is transferable with donor bone marrow; and the persistence of donor hematolymphoid cells is a prerequisite for continued specific nonreactivity.

Human studies using preconditioning with monoclonal antibody therapy and *delayed* infusion of donor bone marrow given several weeks after kidney transplantation have shown some promise, but have fallen short of expectations.[37] Although rejection was not completely avoided and all of the recipients were not made tolerant, it was encouraging that rejection and chimerism were less frequently encountered together in long-term survivors.

After successful bone marrow transplantation in humans, the entire immune system of the donor is transferred into the recipient[38–40] similar to the fully chimeric model of Main and Prehn.[22] Since an appreciable risk of GVHD is present at least early after transplantation, immunosuppressive therapy is needed. Many patients, however, who successfully engraft with donor stem cells become tolerant of the recipient tissues and can be completely weaned from immunosuppressive drugs without adverse consequences.[38–40]

AN OBSERVATION LINKING TWO AREAS OF RESEARCH

There have been few attempts to confront or reconcile the apparent paradox, or dichotomous functions, of donor leukocytes in transplantation biology.[41] First, a description was given of how they have been incriminated as initiators of rejection, and thus deleterious to allograft survival. Then, ample evidence has been provided that donor leukocytes can be used to induce specific tolerance to an allograft. Few investigators may even have recognized that a paradox existed, because it has been widely assumed that passenger leukocytes in a solid-organ allograft are fundamentally different from bone marrow cells. The former are thought to consist mostly or exclusively of mature cells, such as DCs or mature T cells, which have a limited life span. The latter are known to contain a much larger fraction of progenitor cells.

However, one need only to view a few clinical liver allograft biopsies with extramedullary hematopoiesis to realize that immature donor cells are not infrequently transplanted with an organ. More important, the recent finding that donor leukocytes can survive in recipient tissues for decades after a solid organ allograft[42–49] certainly invalidates the assumptions that passenger leukocytes are terminally differentiated cells or are rapidly destroyed. Above all, knowing that donor leukocytes from an allograft organ can survive in the recipient long after transplantation has forced us to realize that the role of passenger leukocytes is more complex and richer than we had ever imagined. Recently, our laboratories have focused considerable attention on the dynamics of passenger leukocyte trafficking and survival.[50, 51]

As noted earlier, DCs are not the only passenger leukocytes, as commonly assumed. There are mature donor T cells that are capable of attacking the host. Mature donor B cells could possibly deliver a tolerogenic signal.[52] Donor hematolymphoid progenitor cell populations are theoretically capable of producing progeny in the recipient.[53] Because each organ differs in terms of its profile of passenger leukocytes, the recipient immune system "sees" a different sum total of immunologic stimuli with each allograft. How each of these donor cell populations interacts with the recipient immune system when the recipient also is simultaneously faced with all of the other donor cell populations certainly highlights the complexity involved in transplantation immunology.

Nevertheless, details of the early emigration patterns of passenger leukocytes from allografts and the participating cell population were recently worked out in some detail in experimental small animal models.[50, 51] Within hours after transplantation leukocytes begin to traffic into and out

of the allograft, following hematogenous migratory routes that are independent of allogenic barriers.[54–57] Because of the genetic disparity, however, donor cells can easily be traced. Donor T and B lymphocytes and DCs can be found in the splenic marginal zone–periarterial lymphatic sheath (PALS) interface and in the PALS a few hours after transplantation.[50, 51] Within days, they appear in the cortex and then in the paracortex of peripheral lymph nodes and the thymic medulla. In an allogenic environment, both donor and recipient cells begin proliferating[50, 51] within days of mixing with each other, the same as in an MLR in vitro.

If no immunosuppression is given to the recipient, a brisk proliferative response develops and the peripheralized donor cells are completely destroyed several days *before* the allograft fails from rejection.[50, 51] If, however, the recipient is even transiently immunosuppressed, the proliferation is quieted, *but not eliminated*. Under the umbrella protection of immunosuppression, a month or so after transplantation the donor leukocytes rapidly disseminate to recipient lymphoid tissues and then to extralymphatic sites such as the skin, intestine, and other visceral organs.

At these sites, the donor leukocytes come to reside in the same anatomic locations as phenotypically identical recipient counterparts.[50, 51] For example, donor DCs can be found in the thymic medulla and the periarterial lymphatic sheath of the spleen; T cells in the paracortex of lymph nodes; B cells in the marginal zone of the spleen; and macrophages and DCs in the skin. It appears as though a fragment of the donor immune system had been transplanted along with the allograft, and had become incorporated into the larger network of the recipient.[41]

If immunosuppressive therapy is withdrawn a month or so after liver transplantation in the Lewis (LEW)/brown Norway rat (BN) strain combination, the number of donor cells slowly declines to very low levels. But even these few cells can survive for up to 200 to 300 days and probably for the lifetime of the animals. In humans, donor hematolymphoid cells have been detected decades after transplantation.[42–48]

The number and phenotypic profile of donor cells surviving long-term depend to some degree on the source of the donor cells. Murase et al.[58] recently showed that BN recipients of LEW liver or bone marrow allografts treated with FK506 for only 4 weeks showed low-level (<0.01%) multilineage chimerism 100 days later. Heart, kidney, thymocyte, peripheral blood, and splenocyte recipients treated in the same fashion were devoid of donor cells at this time. Moreover, microchimeric rats accepted a subsequent allograft at 100 days from the same donor without immunosuppression, whereas rats devoid of microchimerism eventually rejected (some chronically) a subsequent allograft from the same donor. Thus the microchimerism after solid organ (liver) transplantation produced the same results as with the bone marrow. The ability of a liver allograft to produce and sustain hematolymphoid microchimerism may, in part, explain the resistance of liver allografts to chronic rejection. Persistence of donor cells may also have other effects on the recipient immune system, such as transference of delayed-type hypersensitivity responses[42, 48] or even resistance to certain diseases.

The same ends can be accomplished without any immunosuppression in mice.[51] Mouse liver allografts are spontaneously accepted with-

out immunosuppression and produce long-lived (at least >200 days) multilineage hematolymphoid chimerism in the recipient. Like the rats that were transiently treated, these untreated mice also are able to accept subsequent allografts from the same donor without immunosuppression. Normally, the extrahepatic organs would be rejected.

That donor leukocytes can survive in the recipients of solid organ allografts, even after immunosuppressive drugs have been withdrawn, has provided the information to reconcile the apparent paradox or dichotomous role of passenger leukocytes in transplantation biology. As described earlier, the persistence of hematolymphoid chimerism is associated with allogenic tolerance; and we have shown that donor hematolymphoid cells can persist in the recipients of solid-organ allograft recipients.[42–47, 49] Moreover, even human bone marrow allograft recipients that were thought to be full chimerics still retain a microchimeric representation of the recipient—a mirror image of the organ allograft recipients.[59, 60] We therefore have hypothesized that in principle, hematolymphoid chimerism occurring after solid organ transplantation is the same as that observed after bone marrow transplantation.[41, 42] Under both circumstances, the hematolymphoid chimerism is responsible for the development of subsequent donor-specific acceptance of allograft without immunosuppression. Exactly how this happens is currently a matter of speculation and the focus of much research. Stimulation of autoreactive regulatory cells,[41] veto cell mechanism,[35] GVH reactivity,[47, 48, 61] peripheral anergy, and other mechanisms are currently being explored.

Unfortunately, although hematolymphoid chimerism appears to be a necessary condition, alone it is not sufficient for the induction of tolerance.[41, 42, 45, 47, 48, 61] The final outcome appears to depend on *interactions* between the two different populations of hematolymphoid cells, and whether a stable equilibrium between destruction of donor cells and the birth of new ones can be achieved. In some instances, the presence of donor hematolymphoid cells will spontaneously decrease responsiveness of the recipient to the point where immunosuppression is no longer needed. In other instances, first the donor leukocytes and eventually the allograft would be destroyed without continual immunosuppression. A similar situation exists in neonatal mice chimeras.[62, 63] In this murine system, the stability of chimerism and thus the ability to accept donor allografts is dependent on the strain combinations used. Some are extremely stable and tolerant of allografts, whereas others have an inherently unstable relationship with donor cells and allografts. They quickly reject both when provoked by immunologic stimuli.

TEST OF A HYPOTHESIS

The long-term persistence of donor cells; the multilineage character of the chimerism; the ability of the bone marrow cell populations to induce allograft acceptance without immunosuppression; and the commonality of the liver and the bone marrow, all point toward engraftment of a small number of progenitor or stem cell populations being responsible for the microchimerism observed after solid organ transplantation. A major theoretical barrier to this hypothesis has apparently been overcome by showing that the dogma of "making space" for bone marrow engraftment by

TABLE 1.
Current Graft Function and Immunosuppression Profile of Patients Who Received Simultaneous Donor Bone Marrow and Organ Transplantation

			Graft Function			Immunosuppression		
Case No.	Allografts	Postoperative Day	Bilirubin [mg/dL]	Creatinine [mg/dL]	C Peptide [pmol/mL]	FK506 [mg/day]	Steroid [mg/day]	Azathioprine [mg/day]
1	Liver + pancreatic islets	145	0.6	—	0.83	10	05.0	00
2	Liver	179	0.4	—	—	03	00.0	00
3	Liver	198	0.5	—	—	12	00.0	00
4	Liver	218	0.9	—	—	08	00.0	00
5	Liver	241	0.5	—	—	06	10.0	00
6	Liver	336	0.7	—	—	03	00.0	00
7	Kidney	108	—	1.2	—	20	15.0	00
8	Kidney	124	—	1.2	—	10	07.5	00
9	Kidney	190	—	1.4	—	18	02.5	00
10	Kidney + islets	239	—	1.9	0.58	07	12.5	00
11	Kidney + islets	241	—	1.6	0.13	28	05.0	75
12	Kidney	295	—	1.6	—	20	00.0	00
13	Kidney	298	—	2.0	—	16	00.0	00
14	Kidney	379	—	1.2	—	04	00.0	00
15	Kidney	431	—	1.7	—	09	00.0	00
16	Heart	146	Good cardiac function			08	20.0	00

TABLE 2.
In Vitro Immune Status and the Detection of Donor Cells in the Combined Bone Marrow and Whole-Organ Recipients

Case No.	Allografts	HVG* (POD)	GVH† (POD)	Donor-Specific MLR Response‡ 1% (POD)	Detection of Donor Cells		
					POD§	FACS‖ (%)	PCR (cPCR)¶
1	Liver + pancreatic islets	15,86	None	34% (85)	82	1.7	+
2	Liver	7,22	None	36% (108)	108	1.8	+
3	Liver	None	54	6% (120)	146**	1.9	+ (1.0%)
4	Liver	None	21,74	87% (145)	167	<0.5	+
5	Liver	33	None	Low responder††	175	5.0	+
6	Liver	24	None	15% (265)	265	NF‡‡	+
7	Kidney	None	None	59% (48)	48	1.7	+
8	Kidney	None	None	5% (72)§§	19	NF‡‡	NF‖‖
9	Kidney	None	None	70% (113)	120	1.9	+
10	Kidney + islets	41,66	None	5% (166)	133	1.4	+

11	Kidney + islets	16	None	50% (168)	171	3.0	+
12	Kidney	None	None	22% (225)	225	0.6	NF¶¶
13	Kidney	16	None	18% (177)	232	NF‡‡	+
14	Kidney	16	None	26% (68)***	315	<0.5	+
15	Kidney	None	None	NF[l]	367	NF‡‡	+ [0.5%]
16	Heart	12-40‖‖	None	130% (65)	68	1.5	+

*HVG = host-versus-graft reaction (rejection); POD = postoperative day.
†GVH = graft-versus host reaction.
‡Percentage of donor-specific mixed lymphocyte reaction (MLR) responses as compared to third party on the last sample tested.
§Last postoperative day tested.
‖FACS = fluorescence-activated cell sorter.
¶PCR (cPCR) = polymerase chain reaction (complementary PCR).
**The samples for PCR and cPCR were obtained on POD 128.
††Cells did not respond to any stimulation in vitro for up to POD 134.
‡‡NF = not feasible; cross-reactive antibodies.
§§No change in donor-specific responses before and after transplant.
‖‖Not feasible; no major histocompatibility class (MHC) class II mismatch.
¶¶Not feasible; no adequate donor spleen cells.
***Single rejection episode, gradually resolving from grade 3A (multifocal moderate acute cellular reaction [ACR] on POD 12 to grade 1B (diffuse mild ACR) on POD40. All subsequent biopsies were negative.

lethal irradiation is probably not valid.[53] Thus, besides replacement of defective parenchymal cell functions, an allograft brings with it the seeds of the donor immune system. Lu et al.,[64] for example, formally showed the presence of numerous DC progenitors in the normal mouse liver, and other studies, have shown that CD34+ hematolymphoid cells reside in normal adult human livers.[65] Moreover, progenitor cell populations are normally found in the peripheral circulation. It is not too much of a fantasy, therefore, to suggest that progenitor cell populations exist in every solid-organ allograft, although clearly, organs like the liver probably have a greater complement of such cells.

If, however, immature passenger leukocytes are beneficial to solid-organ allograft survival, augmenting the natural emigration out of an allograft by *simultaneous* infusion of donor bone marrow should ultimately result in a greater number of recipients who are eventually drug-free. Other protocols mentioned above often rely on delayed infusion. Our augmentation hypothesis is currently being tested at the University of Pittsburgh in a group of patients who have undergone either kidney, liver, heart, or pancreatic islets transplantation with the *simultaneous* infusion of 3×10^8 cells/kg body weight of unfractionated donor bone marrow.[66] These recipients were not preconditioned in any way and no antilymphocyte globulin induction therapy was used. Instead, they received the standard two-drug immunosuppressive cocktail of FK506 and steroids. The patients are currently being prospectively followed for the presence of hematolymphoid chimerism, graft rejection, and in vitro evidence of donor-specific alloreactivity. The results to date are shown in Tables 1 and 2.

At this time, it is too early to determine whether our hypothesis is correct, but several points have become clear. In contrast to small experimental animal models, the presence of hematolymphoid chimerism is *not synonymous* with protection from rejection or GVHD, particularly in the early posttransplant period. Both serious rejection reactions and minor GVHD have occurred in this population, simultaneously, with the detection of peripheral blood chimerism. Secondly, infusion of donor bone marrow under the conditions employed is relatively safe. It does not appear to result in increased or unmanageable rejection. Moreover, stable hematolymphoid chimerism has been observed for as long as 14 months after transplantation (the longest patient follow-up), all without prior conditioning of the recipient. The degree of chimerism in patients with perioperative bone marrow augmentation is orders of magnitude greater than that observed after solid organ transplantation alone.[66]

The aim of the aforementioned protocol is to reduce the number of patients who require lifelong nonspecific immunosuppression after solid-organ transplantation. Drug-free allograft acceptance and freedom from GVHD for everyone who receives an allograft with bone marrow augmentation is an unreasonable expectation. The potential benefits will be long-term and likely will be dictated by the genetic constitution of the recipient and donor similar to the strain dependency observed in small animal models. It is unlikely, however, that compatibility will segregate along the lines of classic MHC matching currently pursued in clinical transplantation.

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